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Transformation of Rat Intestinal Epithelial Cells by Over-expression of Rab25 is Microtubule Dependent

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Abstract

Little research has addressed the role of membrane trafficking and recycling in the regulation of the transformed phenotype of neoplastic cells. The small GTPase Rab25 is an epithelial-specific modulator of membrane recycling. Recent studies have demonstrated that Rab25 expression is up-regulated in a number of epithelial cancers and over-expression may increase the aggressive phenotype of certain cancers. We have utilized the non-transformed RIE cell line to examine the influence of Rab25 on transformation. Over-expression of Rab25 in RIE cells leads to morphological transformation as well as growth in soft agar, tumor formation in nude mice, disruption of integrin-based focal adhesions and alteration in modified microtubule subsets. Although the predominance of recent cancer research has focused on the manipulation of the actin-based cytoskeleton, recycling trafficking relies on microtubules. Transformation of RIE cells through over-expression of Rab25, but not with H-Ras^{V12}, was reversed by inhibitors of microtubule polymerization. These results suggest that up-regulation of Rab25 in RIE cells leads to microtubule-dependent transformation. Thus, depolymerization of microtubules may be a potent therapeutic target for cancer therapy through the reversal of the invasive phenotype of certain cancer cells.

Keywords

Rab25; β1-integrin; paxillin; microtubules; indibulin; nocodazole

Introduction

Vesicle trafficking events regulate the composition of the cell surface through the modulation of exocytotic, endocytotic and membrane recycling pathways. The Rab proteins are a sub-family of the Ras superfamily of small GTPases and are known to regulate specific

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trafficking pathways within cells (Armstrong 2000). Rab25 is a member of the mammalian Rab11 family, which also consists of Rab11a and Rab11b (Bhartur et al. 2000). The Rab11 family members mainly differ within a 20 amino acid stretch near their carboxy-termini. While Rab11a (Goldenring et al. 1996) and Rab11b (Lai et al. 1994) are ubiquitously expressed, Rab25 is only expressed in epithelial cells (Goldenring et al. 1993). In polarized MDCK cells, both Rab11a and Rab25 are associated with the apical recycling endosome and the trafficking of polymeric IgA, but not transferrin (Casanova et al. 1999; Leung et al. 2000; Wang et al. 2000). While over-expression of Rab11a or Rab11aS20V (the constitutively GTP bound mutation) enhanced trafficking (Ullrich et al. 1996; Wang et al. 2000), the over-expression of Rab25 or Rab25S21V (the constitutively GTP bound mutation) inhibited apical recycling and transcytosis of polymeric IgA (Casanova et al. 1999; Wang et al. 2000), indicating that Rab25 may act antagonistically to Rab11a. Nevertheless, recent work indicates that Rab25 specifically regulates a sorting step in the apical to basolateral transcytosis of IgG by the Fc receptor (Tzaban et al. 2009).

In recent years, several investigations have reported up-regulation of Rab25 expression in a variety of epithelial cancers including prostate, bladder, liver, breast and ovary. Utilizing a series of cell lines generated from the C3/(1)/Tag-Pr transgenic mouse at different stages of prostate cancer, Calvo et al demonstrated that Rab25 was up-regulated in relation to prostate tumorigenicity (Calvo et al. 2002). Mor et al documented that Rab25 was significantly up-regulated in transitional cell carcinoma of the bladder (Mor et al. 2003), while Rab25 is highly expressed in cholangiohepatoma compared with low expression in normal liver samples (He et al. 2002). Wang et al found Rab25 expressed in breast tumor cell lines, including one that was metastatic (Wang et al. 2002), while Cheng et al showed that Rab25 was up-regulated in both breast and ovarian cancer and that its expression correlated with the aggressiveness of the tumor and inversely with survival (Cheng et al. 2004). A third group (Cheng et al., 2006) reported the loss of expression in breast cancer cell line, RAO-3, suggesting that both over-expression and loss of Rab25 can transform cells. These results have led to the suggestion that loss of Rab25 in the context of tumors lacking estrogen receptors leads to neoplasia in breast cancer. Additionally, we have recently found that Rab25 loss is associated with increased susceptibility to intestinal carcinogenesis in mouse models (Nam et al. 2010). Thus, either over-expression or under-expression of Rab25 may lead to transformation based on the context for altered trafficking pathways present in particular cell systems.

All of these previous studies were performed on Rab25 over-expression and knock-down in transformed cell lines (Cheng et al. 2006; Cheng et al. 2004; Cheng et al. 2005; Fan et al. 2006). In the present investigations, we have evaluated the influence of Rab25 on cell transformation in the non-transformed Rat Intestinal Epithelia (RIE) cell line. Our results show that over-expression of Rab25 in RIE cells leads to morphological transformation. Importantly, we find that the transformed phenotype can be reversed by treatment with microtubule polymerization inhibitors. Thus, treatment with microtubule polymerization inhibitors may represent an important therapeutic modality for epithelial tumors with over-expression of Rab25.

Materials and Methods

Cell line production and culture

The pEGFP-Rab25 (Casanova et al., 1999) vector was stably transfected into rat intestinal cells (RIE) using Effectine (Qiagen, Valencia, CA) according to the manufacture's protocol. Clones were selected with 300 μg/ml of hygromycin and surviving clones were evaluated for GFP-Rab25 expression. Cells were maintained with 150 μM/ml of hygromycin. The RIE-iRas cell line with over-expression of Ha-Ras^{Val12} under transcriptional control of the

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Lac operon has been previously described (Sheng et al., 2000). Both cell lines were grown in D-MEM with 10% FBS and antibiotics. The iRas cells were treated with 1mM IPTG 18–24 hours prior to use.

Immunofluorescence

Cells were plated onto coverslips (RIE 400 000; RR25 and iRas 250 000) and allowed to grow for 3–4 days. Where indicated, cells were treated with 3.3 μM Nocodazole (Calbiochem, Gibbstown, NJ) or 1 μM Indibulin (Zeopharma, Inc. Boston, MA) for 18–24 hrs at 37oC. Cells were fixed in 4% paraformaldehyde in PBS or for tubulin staining MT fix (200 mM Pipes, 2 mM EGTA, 2 mM $MgSO_4$, 0.2% TritonX-100, 60% glycerol pH 7.0) for 20 min. at RT, washed with PBS (OminPure, EMD, Gibbstown, NJ), then extracted and blocked in 10% normal donkey serum (NDS), 0.3% Triton X-100 in PBS. Primary antibodies were diluted in 1% NDS 0.05% Tween20-PBS (PBS-T) for 2 hr at RT, washed with PBS-T then incubated for 1 hr at RT with secondary antibodies, washed with PBS-T then with PBS, rinsed in water and mounted with ProLong (Invitrogen, Carlsbad, CA). The cells were imaged with a Olympus (Center Valley, PA) FV1000, that is maintained in the Vanderbilt Cell Imaging Shared Resource, using the 60X oil immersion lens and 3X optical zoom. Primary antibodies used were hamster anti-β1-integrin (BioLegend, San Diego, CA), mouse anti-α2-integrin (clone 12F1, BD Biosciences, San Jose, CA), mouse anti-paxillin (clone 5H11, Millipore, Billerica, MA), mouse anti-vinculin (clone VIN-11-5), mouse anti-α-tubulin (clone B-5-1-2), anti-acetylated tubulin (clone 6-11B-1), anti-polyglutamylated tubulin (clone B3), anti-tyrosine tubulin (clone TUB-1A2) (Sigma, St Louis, MO) and rabbit anti-FAK (C-20, Santa Cruz, Santa Cruz, CA). Secondary antibodies were Cy-3-anti-mouse or anti-rabbit and cy5-anti-hamster (Multi-label, Jackson ImmunoResearch, West Grove, PA). To visualize actin Alexa647-phalloidin (Invitrogen) was added with the secondaries.

To quantitate the movement of the focal adhesion proteins, the XZ images for the individual channels were divided into thirds with the bottom 1/3 representing the focal adhesion proteins contained in the basal membrane and the top 2/3 indicating mislocalized focal adhesion proteins. Using ImageJ the mean gray value (sum of the gray values of all the pixels divided by the number of pixels) for each area was calculated and the data were presented as the percent of the total grayscale value contained in the top 2/3, indicating amount of focal adhesion protein that is internalized and no longer in the basal membrane.

Soft Agar Assay

One ml of 0.5% agarose in media was placed in the bottom of a 12-well plate. One ml of 0.33% agarose in media with 50 000 cells was layer on top, with a final layer of 0.1 ml of media. Where indicated the following treatments were added into the top agarose layer and added upon refeeding, 0.33 μM nocodazole, 0.1 μM indibulin, 1 mM IPTG (iRas cells only). The colonies were allowed to grow for 2 weeks, then stained with Thiazolyl Blue Tetrazolium Bromide (Sigma), 0.5 mg/ml in PBS for 1 hr at 37oC and analyzed on a Gelcount Colony Counter (Oxford Optronix, Oxford, UK) in the Vanderbilt Epithelial Biology Center Imaging Resource.

FACS analysis

RIE or RR25 cells were plated in 10 cm dishes, the cells were trypsinized, quenched with media containing 10% fetal calf sera and washed with cold PBS. All incubations were performed at 4oC on a rotisserie rotator and all solution were ice cold. The cells were blocked in 10% NDS for 30 min., then incubated with or without hamster anti-β1-integrin (BioLegends) for 1 hour. Cells were washed with PBS, then incubated with cy3 anti-hamster (Jackson ImmunoResearch) for 1 hr and washed with PBS. The cells were analyzed on a 5 laser BD LSR-II configured with (355nm, 405nm, 488nm, 535nm, and 633nm excitation

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lines). The phase images of the colonies were taken with the 40X lens of an inverted Olympus scope.

Tumor Growth in Nude Mice

One million RIE cells or RR25 cells were suspended in sterile phosphate buffered saline and injected subcutaneously into opposite flanks of 6 nude mice. The tumors were resected and measured eight weeks after injection. All animal protocols were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Results

Over-expression of GFP-Rab25 caused a microtubule dependent morphology change

To assess the effects of Rab25 on intestinal cells, we established an RIE cell line stably over-expressing GFP-Rab25 (RR25 cells). While the parental RIE cells exhibited a flat, "fried egg" morphology, the RR25 cells were more spindle-shaped and lost some contact inhibition, similar to, but not as drastic as the transformed morphology observed in another RIE cell line expressing inducible H-Ras^{Val12} (iRas cells)(Sheng et al. 2000) (Figure 1A; control). Three other RIE cell lines stably over-expressing EGFP-Rab25 all showed a similar transformed phenotype. No transformed phenotype was observed in an RIE cell line stably over-expressing the closely related family member, GFP-Rab11b (Supplemental Figure 1) although the cells did appear a little smaller then the parental RIE cells.

Given the importance of microtubules to Rab25-dependent trafficking (Casanova et al. 1999), we examined whether incubation with inhibitors of microtubule polymerization could affect the transformed phenotype elicited by Rab25 over-expression. Treatment with nocodazole, which caused depolymerization of many of the microtubules (Figure 5), reversed the morphological changes induced in RR25 cells (Figure 1A, nocodazole). In addition, treatment with indibulin, a compound reported to only effect non-acetylated microtubules (Wienecke and Bacher 2009), also reversed the morphological transformation in RR25 cells (Figure 1A, indibulin). Interestingly, neither nocodazole nor indibulin had any effect on the transformation of the RIE cells over-expressing H-Ras^{V12} (Figure 1A). Previous studies have suggested that H-Ras^{V12} induced transformation is mediated by actin-based mechanisms involving Rac activation (Lee & Helfman, 2004). Neither cytochalasin D nor latrunculin B treatment reveresed the cellular morphology or β1-integrin localization (Supplemental Figure 2S). Treatment with taxol did not reverse the transformed morphology of either the RR25 or iRas cells (results not shown). These results demonstrated that two different cytoskeletal-based mechanisms could lead to transformation of RIE cells.

Over-expression of Rab25 promoted tumour growth in nude mice and colony formation in soft agar

To investigate further whether this transformed phenotype was just morphological or whether a functional transformation of the RIE cells occurred with the GFP-Rab25 over-expression, we evaluated the ability of the RR25 cell line to form tumors in nude mice and colonies in soft agar. Non-transformed parental RIE cells do not grow as tumors in nude mice. However, when the RR25 cells were implanted into nude mice, 5 of the 6 mice developed a tumor greater than 5 mm in size at the subcutaneous injection site eight weeks after injection of one million cells. No tumors developed on the opposite flanks where the parental RIE cells were injected (Figure 1B). Parental RIE cells also did not form colonies suspended in soft agar while both the RR25 and the iRas cells were able to form colonies (Figure 1C and D). These results indicated that over-expression of Rab25 led to functional transformation of RIE cells.

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We next examined whether the behavior of transformed RIE cells in soft agar was also affected by disruption of microtubules. Both nocodazole and indibulin were able to reverse significantly soft agar colony formation by RR25 cells, but neither compound had any effect on the ability of iRas cells to form soft agar colonies (Figure 1D). Thus, both the transformed morphology and the ability of the RIE cells over-expressing GFP-Rab25 to grow in soft agar were dependent upon intact microtubules. These results indicate that a change in microtubule-based trafficking could lead to transformation.

Over-expression of Rab25 altered the plasma membrane localization of β1-integrin

Members of the Rab11 family have been implicated in trafficking of integrins that affect both cell migration (Jones et al. 2006; Powelka et al. 2004) and tumor invasion (Chia and Tang 2009; Tang and Ng 2009; Yoon et al. 2005), and specifically Rab25 has been implicated in β1-integrin trafficking in an ovarian cancer cell line (Caswell et al. 2008; Caswell et al. 2007). We therefore investigated whether perturbation of β1-integrin localization might account for transformation of GFP-Rab25 over-expressing RIE cells. In the parental RIE cells, the majority of the β1-integrin was located in the basal plasma membrane (Figure 2A, row 1). In the GFP-Rab25 or the H-Ras^{V12} over-expressing RIE cell lines, the β1-integrin was displaced from the basal plasma membrane to an internal compartment, (Figure 2, compare the XZ images of row 2 and 3 with row 1), as well as to regions in the apical surfaces of the cell. Unlike Caswell et al (Caswell et al. 2007), we did not observe any co-localization of the GFP-Rab25 and β1-integrin in the RR25 cells (Figure 2A, row 2). The β1-integrin in the iRas cells appeared more internally dispersed than in the RR25 cells (Figure 2A, compare rows 2 and 3). To quantitate loss of β1-integrin from the cell surface, we utilized FACS analysis on non-permealized cells. As seen in Figure 2B, the RR25 cells had 50% less β1-integrin on their cell surface compared to the parental RIE cells. So, while we did not observe co-localization of β1-integrin with over-expression of GFP-Rab25 in RIE cells, we did observe an alteration in β1-integrin localization from the basal plasma membrane to an internal compartment.

Over-expression of Rab25 altered the composition of focal adhesions

To investigate whether the whole focal adhesion complex or just β1-integrin was disturbed by the over-expression of GFP-Rab25, we then evaluated four other components of the focal adhesions, α2-integrin (Figure 3A), focal adhesion kinase (FAK)(Figure 3B), vinculin (Figure 3C) and paxillin (Figure 4 control). The α2-integrin was also mislocalized to an internal pool in the RR25 cells (RIE 11%; RR25 32%). We observed α2-integrin co-localized with both the plasma membrane β1-integrin in the RIE cells and the internalized β1-integrin in the RR25 cells, indicating that the integrin heterodimer was still intact upon internalization (Figure 3A). While we did observe some internalization of FAK (Figure 3B) the difference between the RIE cells (16%) and RR25 cells (24%) was not statistically significant. However, vinculin (Figure 3C), was significantly internalized from focal adhesions in the RR25 cells. We also stained for the scaffolding protein, paxillin (Figure 4). In the parental RIE cells, the majority of the paxillin was located in the basal plasma membrane (Figure 4A and E control showing only 14% internal). In contrast, in both the RR25 (Figure 4B and E) and iRas (Figure 4C and E) cell lines, over 40% of the paxillin was found in internal compartments. Western blots confirmed that total paxillin was not altered in either the RR25 or iRas cells (data not shown).

Since treatment with either nocodazole or indibulin reversed the transformed morphology of the RR25 cells, we tested whether depolymerization of the microtubules could also reverse the loss of β1-integrin and paxillin from the focal adhesions. As seen in Figure 4, treatment with either nocodazole or indibulin significantly reversed the internalization of both paxillin and β1-integrin returning their predominant localization to the basal plasma membrane in the

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RR25 cell line (Figure 4B, 4D, 4E). However, no effects of either nocodazole or indibulin treatment were observed in the iRas cell line (Figure 4C, 4D, 4E). Neither the indibulin nor the nocodazole had a discernible effect on the parental RIE cells (Figure 4A, 4D, 4E). While treatments in all of these studies were performed for 18–24 hours, similar effects were observed when cells were treated with 10-fold higher doses of the microtubule inhibitors for 4 hours (data not shown). These results support the concept that the transformation caused by over-expression of Rab25 has a different underlying mechanism from transformation by over-expression of H-Ras^{V12}.

Changes in microtubule subsets

Since the transformation phenotype is dependent upon microtubule stability, we examined the effect that over-expression of GFP-Rab25 has on either acetylated or tyrosinated subsets of microtubules. Figure 5 displays staining for alpha–tubulin as an indicator of general microtubule distribution. In subconfluent, untreated RIE cells, the majority of the microtubules were distributed in a meshwork fanning out from a central nidus characteristic of the pericentrosomal region. When the parental RIE cells were treated with nocodazole microtubules were markedly decreased except in the peri-centrosomal region, while treatment with indibulin produced a more elaborate and finer meshwork of microtubules throughout the cell (Figure 5 top). In the GFP-Rab25 expressing cells (Figure 5 bottom) the microtubules were distributed in long bundles within the cell and this bundling could be reversed to a finer meshwork by treatment with either nocodazole or indibulin. A similar staining pattern was seen when confluent RIE cell lines were stained (supplemental Figure 5S).

The pattern seen when the cells were stained for tyrosinated tubulin was similar to that seen when the cells were stained for α-tubulin. In the sub-confluent RIE cells a meshwork of tyrosinated microtubules was observed (Figure 6 top) while tyrosinated microtubules formed long bundles and cellular extensions when GFP-Rab25 was expressed (Figure 6 bottom). These bundles returned to a more normal cellular meshwork when the RR25 cells were treated with either nocodazole or indibulin. In confluent cells overexpressing GFP-Rab25 (Supplemental Figure 6S), the staining pattern was similar except that instead of long cells with long bundles of tyrosinated tubulin, the more compacted cells made long extensions across other cells and these extensions mainly contained tyrosinated tubulin.

When the parental RIE cells were stained for acetylated tubulin, the majority of the labeling was found in the pericentrosomal area of the cell and in the primary cilium in sub-confluent cells (Figure 7). In the non-treated RIE cells (Figure 7, top), the acetylated tubulin was mainly in the basal aspects of the cell and in the primary cilium. With nocodazole treatment more acetylated microtubules were observed within the cell as thick short bundles of microtubules as well as in the primary cilium. More acetylated microtubules were also observed in the indibulin-treated cells, but these were distributed in a finer meshwork. In the GFP-Rab25 overexpressing cells (Figure 7, bottom), the acetylated tubulin was present in very short microtubules as well as in the primary cilium. When the RR25 cells were treated with nocodazole, the acetylated microtubules were slightly longer. With indibulin treatment, the acetylated microtubules were present in a tight perinuclear meshwork, branching outwards in the cell as well as in the primary cilium. In confluent cells, the acetylated microtubules did not change drastically either with the expression of GFP-Rab25 or treatment with the microtubule targeted drugs (Supplemental Figure 7S). Interestingly, in both the sub-confluent (Figure 7) and confluent cells (Supplemental Figure 7S), the perinuclear location of the GFP-Rab25 in the indibulin treated cells was associated with the enriched acetylated microtubule network. In the sub-confluent RR25 cells treated with nocodazole, the GFP-Rab25 was located at the ends of the actin-rich processes. These observations indicate that, even though the transformation phenotype of the RR25 cells is

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reversed by both nocodazole and indibulin, the mechanisms of action on microtubule-based trafficking are likely distinct.

Discussion

Recent investigations have noted altered expression of some Rab proteins in tumors. Rab5 and Rab7 were both upregulated and demonstrated a higher degree of membrane association in autonomous thyroid adenomas (Croizet-Berger et al. 2002). Alteration of Rab25 expression has been variously reported as either up-regulated or down-regulated in a number of human cancers (Calvo et al. 2002; He et al. 2002; Mor et al. 2003). In breast cancer the majority of evidence suggests that only a subset of tumors show upregulation of Rab25 (Wang et al. 2002), while Rab25 loss is likely more common in breast cancer, especially those without estrogen receptor expression (Cheng et al. 2006; Cheng et al. 2009; Cheng et al. 2004). Similarly, we have noted recently that Rab25 is generally reduced in human colon cancer and loss of Rab25 can promote intestinal carcinogenesis in mouse models (Nam et al. 2010). Nevertheless, in the RIE cell line utilized in this study, over-expression of Rab25 elicits transformation. Thus, over-expression or under-expression of Rab25 may lead to transformation, based on the context for altered trafficking pathways present in particular cell systems. All of these studies suggest that Rab proteins may influence tumor behavior through their involvement in membrane trafficking.

Here we investigated the effects of over-expression of GFP-Rab25 in a non-transformed epithelial cell line. Over-expression of GFP-Rab25 in a rat intestine epithelia cell line (RIE) altered the morphology of the RIE cells from a normal “fried-egg” morphology into a transformed phenotype with spindling of cells, establishment of long cell extensions and loss of an organized monolayer. The alterations were not just restricted to a morphological shift but reflected a true transformed phenotype. Thus, while parental RIE cells did not develop tumors in nude mice or form colonies in soft agar, the GFP-Rab25 overexpressing RIE cell line did form tumors in nude mice and grew as colonies in soft agar. These characteristics were similar to those seen for RIE cells transformed with dominant-active H-Ras, but Rab25 over-expressing cells were actually even better at forming colonies in soft agar. Thus, Rab25 over-expression can induce a functional transformation of this non-transformed cell line. Interestingly, in a polarized epithelial cell line, Madin-Darby Canine Kidney cells (MDCK), over-expressing Rab25 (Casanova et al. 1999), did not elicit a transformed morphology. Thus the ability of Rab25 to cause cellular transformation is cell type dependent.

Hypoxia-stimulated tumor invasion is dependent on both Rab11 and microtubules and involves an increase of $\alpha_6\beta_4$-integrin on the surface of invading cells (Yoon et al. 2005). Rab11 has also been implicated in β1-integrin trafficking in HeLa cells (Powelka et al. 2004). In an ovarian cancer cell line, Caswell, et al. reported co-localization of over-expressed Rab25 (Caswell et al. 2007) with the over-expression of an effector of the Rab11 family, Rab11-FIP1C (RCP). Rab11-FIP1C (RCP) was internalized with β1-integrin in A2780 cells, an ovarian cancer cell line (Caswell et al. 2008). Caswell et al reported that β1-integrin was displaced from the basal plasma membrane to an internal compartment in GFP-Rab25 over-expressing ovarian cancer cell lines. We also observed displacement of β1-integrin in to an internal compartment but, unlike Caswell, we did not observe β1-integrin and GFP-Rab25 co-localized. This could be due to cell specific differences between the non-transformed RIE cell line we used and the ovarian cancer cell line they used. Caswell et al never showed co-localization of β1-integrin with either endogenous Rab25 (Caswell et al. 2007) or endogenous Rab11-FIP1C/RCP (Caswell et al. 2008). However, while these investigators did observe the immunoprecipitation of over-expresssed Rab25 with β1-integrin, they did not indicate how much of the β1-integrin was co-immunoprecipitated with the over-expressed Rab25. Thus, only a small fraction of the endogenous β1-integrin may be

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associated with Rab25 (Caswell et al. 2007). Unfortunately the only β1-integrin antibody that consistently worked in rat cells does not work for immunoprecipitation or western blotting, so we were not able to investigate if a small amount of the β1-integrin is associated with the GFP-Rab25 in RIE cells. Still, all of the present evidence suggests that alterations in RIE behavior with Rab25 over-expression are likely referable to alterations in trafficking related to integrin targeting or recycling to the plasma membrane.

Importantly, Rab25 over-expression also affected other components of the focal adhesions. While the majority of FAK remained at the plasma membrane vinculin, paxillin and α2-integrin were displaced internally with over-expression of GFP-Rab25. Paxillin was internalized separately from both β1-integrin and GFP-Rab25, but α2-integrin was internalized with the β1-integrin indicating retention of the integrin complex, likely in a vesicle membrane. Since we did not observe any co-localization between GFP-Rab25 and either β1-integrin or paxillin, the over-expression of Rab25 may either alter the trafficking of a common focal adhesion regulator, such as a kinase for paxillin, or influence a secondary trafficking pathway for delivery or endocytosis of adhesion components. One potential scenario could involve GFP-Rab25-dependent effects on the Eps15-homology domain-containing protein 1 (EHD1). Caplan and colleagues have shown that EHD1 can regulate β1-integrin transport, thus affecting focal adhesions (Jovic et al. 2007). Interestingly our group has shown that EHD1 and Rab8a co-localized on membrane tubules (Roland et al. 2007). Rab8a interacts with the motor protein Myosin Vb (Roland et al. 2007) and we have previously shown that Myosin Vb can interact with both Rab11a and Rab25 (Lapierre et al. 2001), so the overexpression of GFP-Rab25 could also be interfering indirectly with the EHD1 pathway by sequestering proteins involved in EHD1-controlled trafficking.

Rab25 might also be exerting its influence on the focal adhesions through the microtubules, known participants in the dynamics of focal adhesions. Kaverina and coworkers showed in live cells that contact of microtubules at focal adhesions regulated their relaxation, leading to speculation that the microtubules are delivering signals for relaxation (Kaverina et al. 1999). While regrowth of microtubules after nocodazole treatment and washout causes disassembly of focal adhesions, this effect is not dependent upon Rho or Rac, but rather on dynamin and FAK (Ezratty et al. 2005). Another group looking at the dynamics of focal adhesions in both the center and periphery of cells concluded that, while actin is important for the tyrosine phosphorylation of paxillin during assembly at focal adhesion, microtubules are important in the regional assembly and disassembly of paxillin at focal adhesions in the periphery of the motile cell (Hu et al. 2006).

Previous work has shown that the membrane recycling controlled by Rab11a is dependent on microtubules (Apodaca et al. 1994; Casanova et al. 1999). Interestingly two microtubule polymerization inhibitors, nocodazole and indibulin, were both able to reverse all of the changes due to the over-expression of GFP-Rab25. However, the mechanisms by which these drugs reverse the morphological changes in RR25 cells must differ. Nocodazole inhibits microtubule polymerization by binding to β-tubulin and preventing formation of one of the two interchain disulfide linkages. When RR25 cells were treated with nocodazole the GFP-Rab25 did not move to the perinuclear location where the recycling system is located in the parental RIE cells but instead, accumulated at the ends of actin extensions. Kolodney and Elson (Kolodney and Elson 1995) showed that disruption of microtubules by nocodazole caused the cells to contract. This contraction was mediated by myosin light chain phosphorylation, indicating a connection between cytoskeleton modulation and myosin. While all subsets of microtubules are affected by nocodazole, indibulin has been reported to depolymerize non-acetylated microtubules, based on its ability to inhibit polymerization of microtubules from calf brain tubulin but not bovine brain tubulin (Wienecke and Bacher 2009). Nevertheless, the microtubule preparations used were not pure

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tubulin and also contained microtubule-associated proteins (MAPs), so the exact target of indibulin action remains to be determined. Interestingly unlike nocodazole, indibulin treatment of the RR25 cells did not relocate the GFP-Rab25 to the ends of actin filament bundles in cell extensions, but instead to the perinuclear area where the recycling system is located. All of these results indicate that the drugs have differential effects on the microtubules needed for Rab25-dependent trafficking. Two actin polymerization inhibitors, cytochalasin D and latrunculin B, did not reverse the transformed morphology of the GFP-Rab25 RIE cells (Supplemental Figure 2S).

Because of the intimate association of recycling membranes with the microtubule cytoskeleton, we examined the effects of nocodazole and indibulin in the context of expression of GFP-Rab25 on modification and maturation of microtubules. Acetylation of tubulin on lysine 40 and the presence of tyrosine on the tubulin carboxyl-terminus is believed to mark stable and dynamic microtubules, respectively (Hammond et al. 2008; Westermann and Weber 2003). Both modifications can cycle on and off microtubules so the ratio between stable and dynamic microtubules within a cell can be altered (Hammond et al. 2008; Westermann and Weber 2003). Acetylation of tubulin is thought to mark stable microtubules, and this stability has implicated the acetylated microtubules in the regulation of some cellular processes, including interacting with and controlling the enzymatic activity of Na^+, K^+-ATPase (Santander et al. 2006). Acetylated microtubules have also been implicated in vesicle trafficking in neurites based on their involvement in the polarized trafficking of the JNK regulator (JIP-1) to neurite tips by kinesin-1 (Kif5) (Reed et al. 2006). However, it appears that binding of other kinesins requires non-dynamic microtubules, since two others groups have shown that only detryrosinated microtubules allow kinesin binding (Dunn et al. 2008; Kreitzer et al. 1999). Cai et al have shown that even within the kinesin family there are different preferences for dynamic and stable microtubules (Cai et al. 2009). We observed changes in both acetylated and tyrosinated tubulin in the RIE and RR25 cells. In sub-confluent RR25 cells, the acetylated microtubules were very short, suggesting that a kinesin or another motor's ability to traffic would be affected in these cells. The return of GFP-Rab25 back to the perinuclear localization following indibulin treatment, but not nocodazole treatment, could indicate a role for an acetylated microtubule interacting motor. Interestingly, work from Bridgman (Bridgman 1999) suggests that although Myosin Va is an actin motor, the state of the microtubules can influence its movement by stranding the motor and its cargo in an area of dynamic, tyrosinated microtubules. Since Myosin Vb motors interact with Rab25 and other members of the Rab11 family (Lapierre et al. 2001; Roland et al. 2007), a Myosin Vb motor may be influenced similarly by the changes in acetylated and tyrosinated microtubules, as seen in the RR25 cells. The changes in tyrosinated microtubules in the treated RIE and RR25 cells may not indicate formation of new microtubules, but rather the re-addition of tyrosine to tubulin dimers by tubulin tyrosine ligase (TLL), prior to their incorporation into microtubule polymers. TLL knock-out mice demonstrate accumulation of detyrosinated tubulin, disorganization of neuronal networks and die perinatally (Erk et al. 2005). Further work indicates that tyrosinated microtubules are important in the organization and pathway of neuronal growth cones potentially though their influence on actin and Rac1 (Marcos et al. 2009). Thus, alterations in microtubule modifications may be influenced by vesicle trafficking and in turn subsets of modified microtubules may provide specific tracks for distinct vesicle trafficking pathways.

Conclusion

In the present studies we have demonstrated that over-expression of Rab25 in RIE cells can lead to transformation of non-transformed RIE cells. In contrast with the previously characterized transformation of RIE cells by expression of activated H-Ras, Rab25-mediated

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transformation was dependent on the intact microtubules and changes in the subsets of modified microtubules could be responsible for these morphological changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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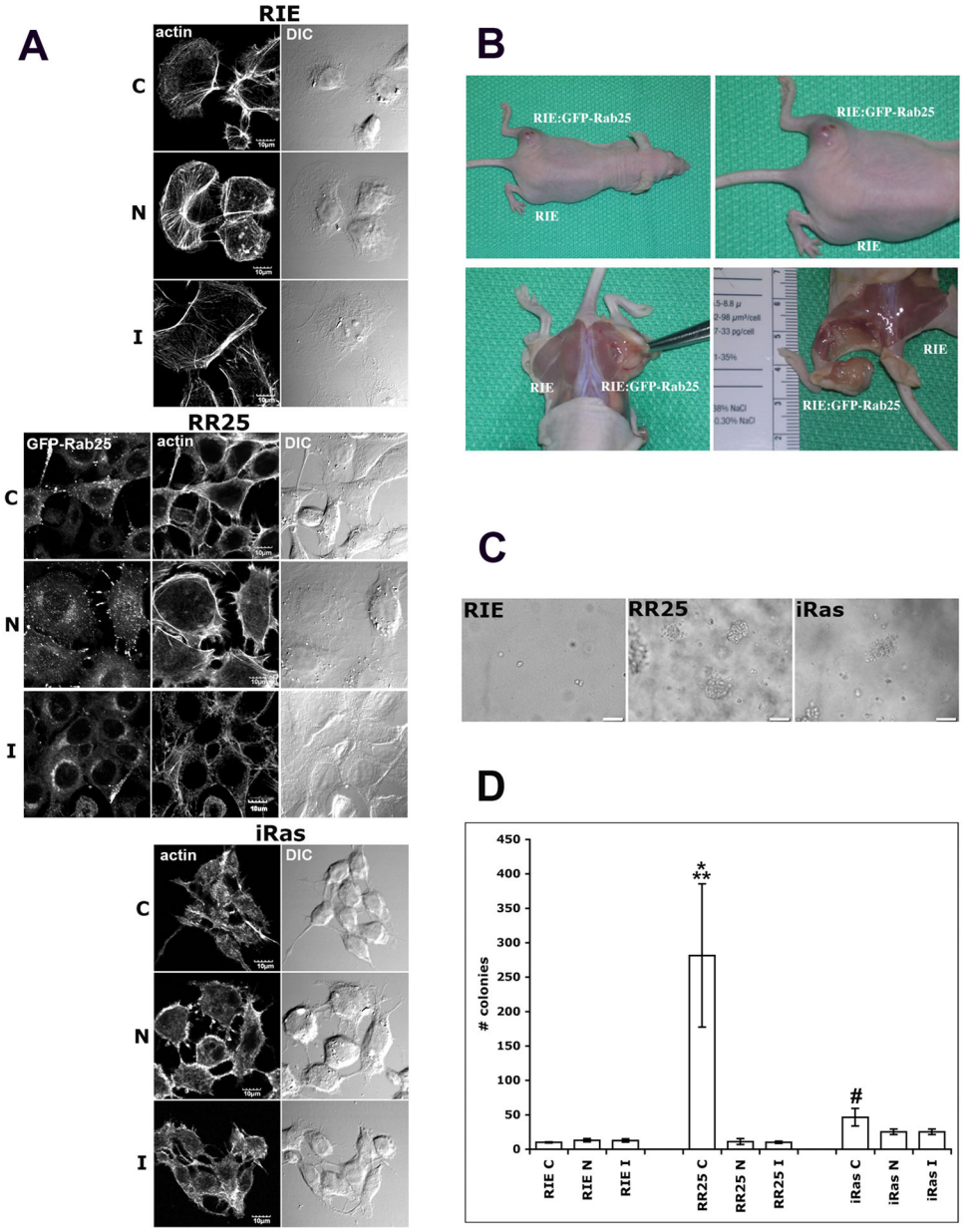

Figure 1. Over-expression of GFP-Rab25 transformed RIE cells in a microtubule dependent manner

A. Parental RIE, RR25 and iRas cells were fixed with paraformaldehyde, stained for actin and imaged for GFP-Rab25, actin and DIC. C=control, N=nocodazole, I=indibulin. Bar=10 μM. **B**. GFP-Rab25 transformed RIE cells, but not the parental RIE cells formed tumors in nude mice. Each panel is a stage in the tumor resection of a representative mouse from 5 of 6 injected mice. **C and D**. iRas and GFP-Rab25 expressing RIE cells, but not the parental RIE cells formed microtubule-dependent colonies in soft agar. Fifty thousand cells were plated in soft agar and allowed to grow for 2 weeks. **C.** Examples of RIE, RR25 and iRas soft agar colonies. **D.** The mean number of colonies from two separate experiments with triplicates at each point. Where indicated the following treatments were added into the top agarose layer and upon refeeding: 0.33 μM nocodazole, 0.1 μM indibulin, 1 mM IPTG (iRas cells only). Error bars are SEM, * p = 0.026 between RIE-control and RR25-control, ** p =

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0.026 between RR25-control and RR25-nocodazole or indibulin treated, # p = 0.018 by *t* test between RIE-control and iRas-control. There was no statistical significance (p = 0.146) between iRas-control and iRas-nocodazole or indibulin treated.

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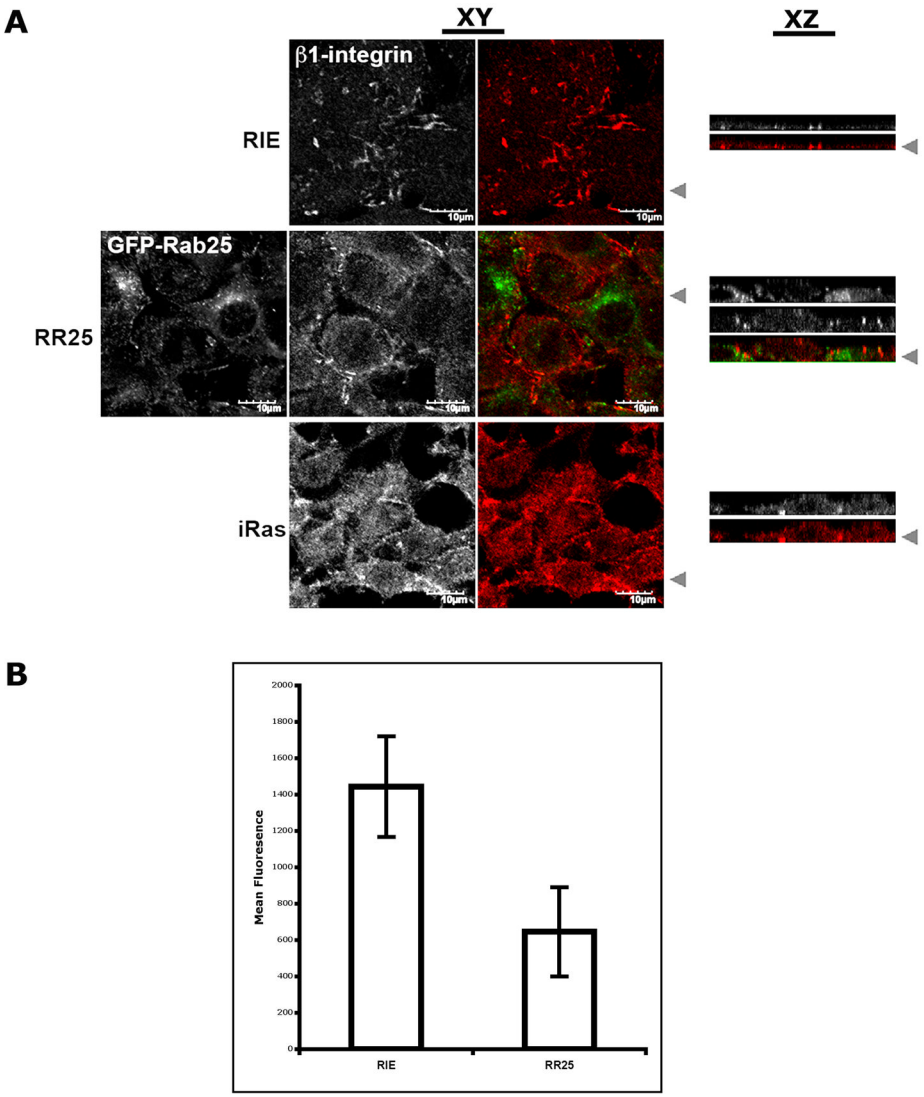

Figure 2. Over-expression of GFP-Rab25 caused mislocalization of β1-integrin

A. Parental RIE, RR25 and iRas cells were fixed with paraformaldehyde and stained for β1-intergrin (red in merged image). The β1-integrin was located in the basal plasma membrane of the non-transformed RIE cells, but in both RR25 and iRas cells β1-integrin was located internally. Arrowheads indicate were the XY and XZ optical cuts were made, bar=10 μm (representative of three separate experiments). **B**. The amount of β1-integrin on the cell surface of non-permeabilized RIE and RR25 cells was quantitated by FACS analysis. Mean fluorescence of β1-integrin on the surface of RIE and RR25 cells of five separate experiments. Error bars are SEM, p = 0.0106 by paired *t* test.

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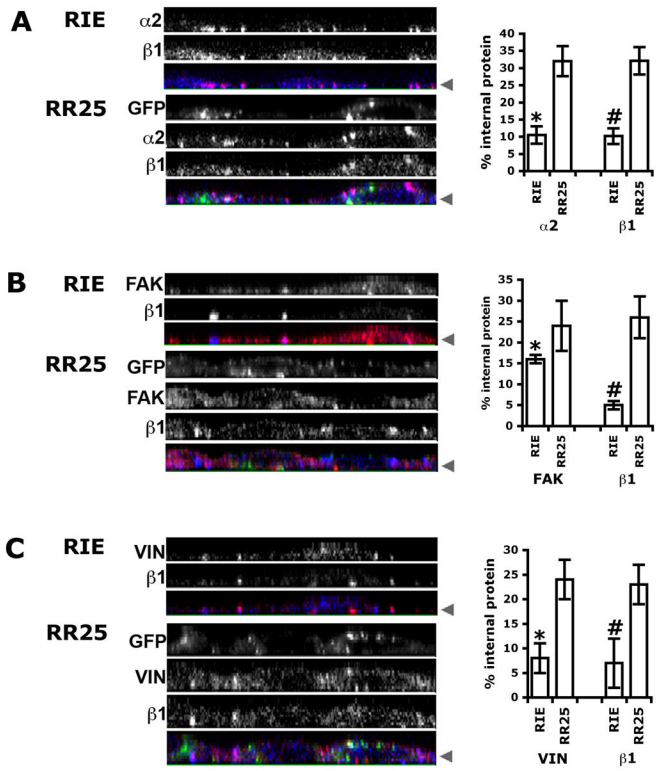

Figure 3. Over-expression of GFP-Rab25 caused mislocalization of α2-integrin and vinculin, but not FAK

In each section a representative XZ series is shown on the left and the quantitation from at least 6 separate images is shown on the right. **A**. Parental RIE and RR25 were co-stained for α2-integrin (red in merge) and β1-integrin (blue in merge). Similar to β1-integrin, α2-integrin was internalized in RR25. * $p < 0.001$, # $p < 0.001$ **B**. Parental RIE and RR25 were co-stained for FAK (red in merge) and β1-integrin (blue in merge). Only a small amount to the FAK internalized. * $p = 0.395$, # $p= 0.05$ **C**. Parental RIE and RR25 were co-stained for vinculin (red in merge) and β1-integrin (blue in merge). Similar to β1-integrin, vinculin was internalized in RR25. * $p = 0.0458$, # $p = 0.0371$ The corresponding XY image is contained in Supplemental Figure 3S and the arrowheads indicate where the XY optical section was taken, bar=10 μm (representative of three separate experiments). Error bars are SEM.

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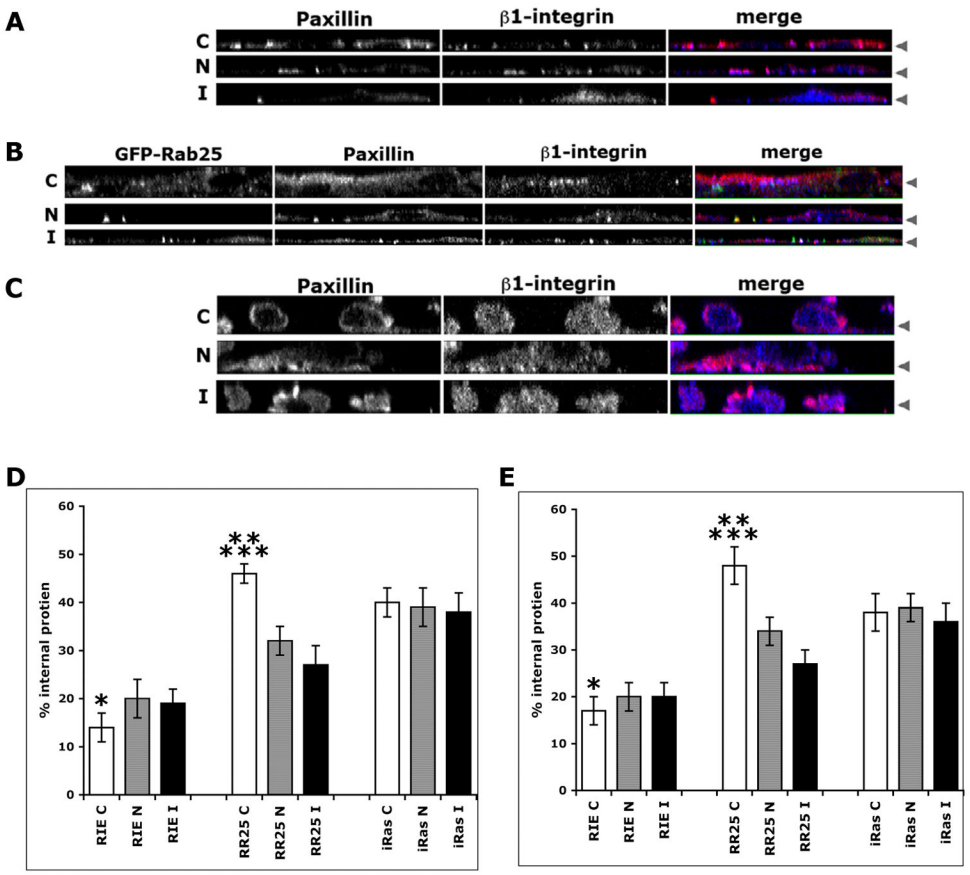

Figure 4. Microtubule depolymerization reversed the mislocalization of β1-integrin and paxillin in RR25 cells, but not in iRas cells

A. RIE, **B**. RR25 and **C**. iRas cells were treated for 18–24 hours with nocodazole (N, 3.3 μM) or indibulin (I, 1 μM) or were left untreated (C), fixed with paraformaldehyde and co-stained for β1-integrin (blue in merge) and paxillin (red in merge). iRas cells were also treated with 1 mM IPTG for 18–24 hours prior to fixing to induce activated H-Ras expression. **D**. Quantitation of β1-integrin from at least 6 separate XZ sections. * $p \leq 0.003$ between RIE-control and either RR25 control or iRas –control, ** $p = 0.0414$ between RR25-control and RR25-nocodazole, *** $p = 0.017$ between RR25-control and RR25-indibulin. **E**. Quantitation of paxillin from at least 6 separate XZ sections. * $p \leq 0.005$ between RIE-control and either RR25 control or iRas–control, ** $p = 0.0207$ between RR25-control and RR25-nocodazole, *** $p = 0.008$ between RR25-control and RR25-indibulin. The corresponding XY image is contained in Supplemental Figure 4S and the arrowheads indicate where the XY optical section was taken, bar=10 μm (representative of three separate experiments). Error bars are SEM.

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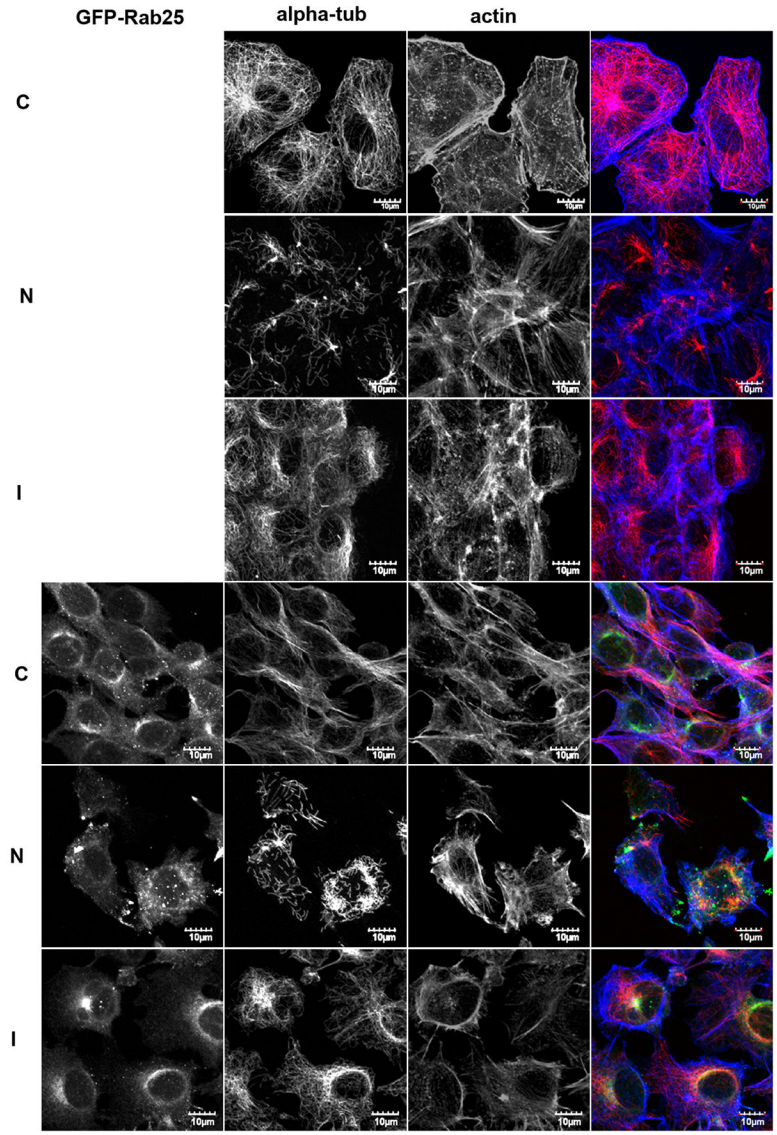

Figure 5. Staining of subconfluent RIE or RR25 cells for α-tubulin showed changes with treatment or GFP-Rab25 over-expression

Subconfluent RIE (top) of RR25 (bottom) cells were untreated (C), treated overnight with nocodazole (N, 3.3 μM) or indibulin (I, 1 μM) then fixed with MT fix and stained for α-tubulin and actin. In merge green is GFP-Rab25, red is α-tubulin and blue is actin. Bar= 10 μM.

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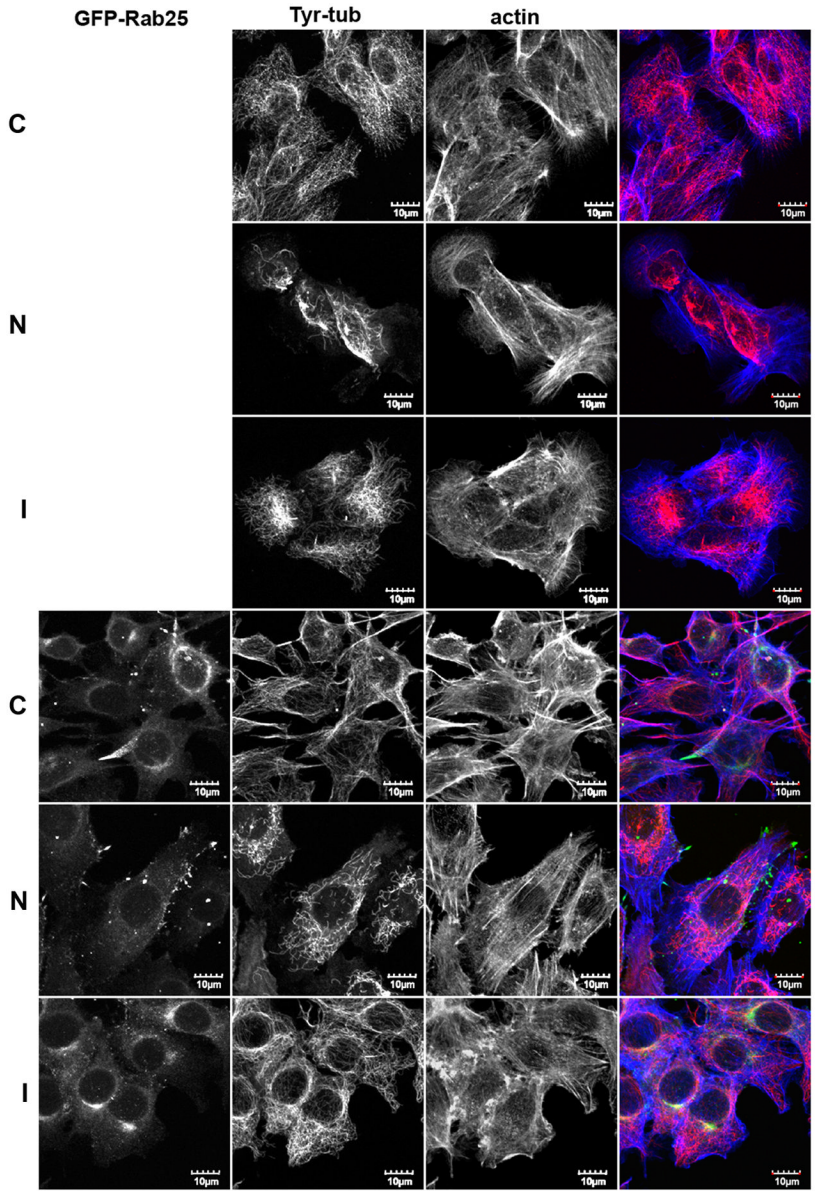

Figure 6. Staining of subconfluent RIE and RR25 cells for tyrosinated-tubulin showed changes with treatment or GFP-Rab25 over-expression

Subconfluent RIE (top) of RR25 (bottom) cells were untreated (C), treated overnight with nocodazole (N, 3.3 μM) or indibulin (I, 1 μM) then fixed with MT fix and stained for tyrosinated-tubulin and actin. In merge green is GFP-Rab25, red is tyrosinated-tubulin and blue is actin. Bar= 10 μM.

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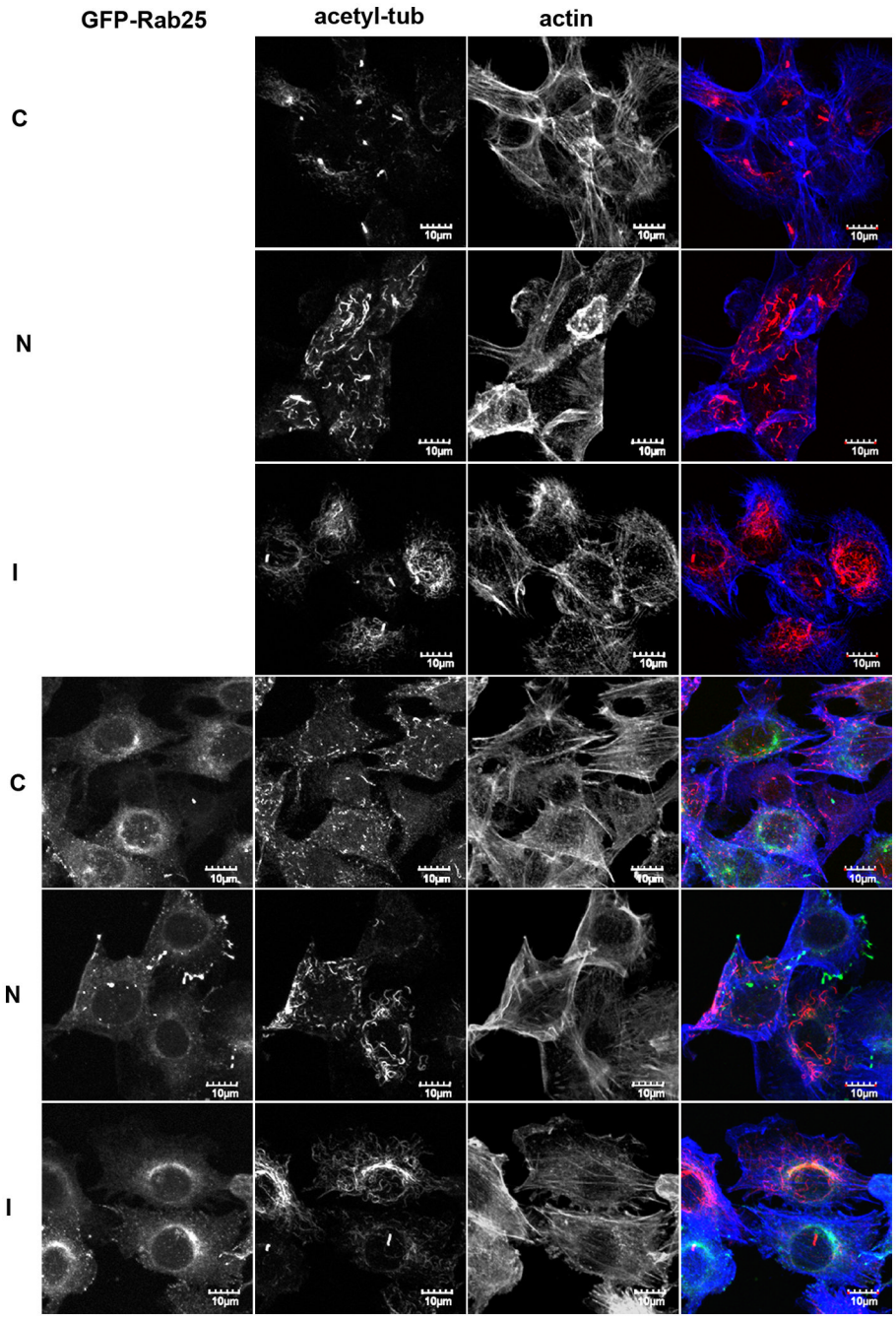

Figure 7. Staining of subconfluent RIE and RR25 cells for acetylated tubulin showed changes with treatment or GFP-Rab25 over-expression

Subconfluent RIE (top) of RR25 (bottom) cells were untreated (C), treated overnight with nocodazole (N, 3.3 μM) or indibulin (I, 1 μM) then fixed with MT fix and stained for acetylated-tubulin and actin. In merge green is GFP-Rab25, red is acetylated tubulin and blue is actin. Bar= 10μM.